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Intron Retention May Regulate Expression of Epstein-Barr Virus Nuclear Antigen 3 Family Genes

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The nuclear antigen 3 family genes (EBNA-3, EBNA-4, and EBNA-6) of Epstein-Barr virus (EBV) are important for EBV-induced immortalization and survival of B lymphocytes. However, little is known about how the expression of these genes is regulated. Each of the EBNA-3, EBNA-4, and EBNA-6 genes consists of two exons separated by a small intron. Reverse transcriptase PCR assays revealed that the vast majority of the EBNA-3, EBNA-4, and EBNA-6 mRNA, expressed in transfected and EBV-infected B cells, retained intron sequences. Northern blot and S1 protection assays confirmed that most of the EBNA-3 mRNA contained intron. Examination of deletion mutants of EBNA-3 indicated that the EBNA-3 protein was not necessary for intron retention and that there was no splicing silencing element encoded in the EBNA-3 mRNA. Cell fractionation and RNA gradient analysis revealed that the unspliced EBNA 3 family mRNAs were transported into the cytoplasm and associated with the polysomes. However, Western blot analysis of FLAG-epitope tagged EBNA-3 gave no indication of the presence of splice variant protein forms of EBNA-3. In contrast, transiently transfected cells expressing EBNA-3 revealed a sixfold increase in EBNA-3 protein expression from the genomic EBNA-3 gene compared to EBNA-3 cDNA. These data show that the intronic sequences can influence EBNA-3 protein expression and suggest that intron retention may provide a means for the fine-tuning of expression of the individual EBNA 3 family genes.

Epstein-Barr virus (EBV) is a human gammaherpesvirus which infects at least 90% of the world's population. EBV is the etiological agent of infectious mononucleosis and is associated with a variety of lymphoid and epithelial cancers including Burkitt's lymphoma (BL), nasopharyngeal carcinoma, and Hodgkin's disease. The oncogenic potential of EBV is reflected in its ability to efficiently transform and immortalize human B cells in vitro. The resulting latently infected lymphoblastoid cell lines (LCLs) express a restricted set of EBV genes encoding six nuclear antigens (EBNA-1 through EBNA-6) and three latent membrane proteins (LMP-1, TP-1, and TP-2). At least two different EBV types exist, A and B (also called I and II), which are classified by sequence divergence in the genes encoding EBNA-2, EBNA-3, EBNA-4, and EBNA-6 (reviewed in reference 18). The pattern of latent EBV gene expression, present in LCLs, is also found during primary B-lymphocyte infection in vivo and in EBV-associated lymphoproliferative disease (39), emphasizing that regulation of latent gene expression is likely to be important in the process of EBV-mediated B-cell transformation, virus persistence, and lymphomagenesis.

The persistent expression of the EBNA 3 gene family products, EBNA-3, EBNA-4, and EBNA-6 (also known as EBNA-3a, EBNA-3b, and EBNA-3c) against negative selective pressure by cytotoxic T cells in vivo (28) is consistent with an important role for these genes. Indeed, it was shown that EBNA-3 and EBNA-6 were essential for B-cell immortalization (40) and that EBNA-4 enhanced the survival of cells prone to undergo apoptosis (35). Expression of the individual genes of the EBNA 3 family resulted in transactivation or repression of viral and cellular genes, suggesting a role as transcriptional regulators (1, 19, 22, 24, 43). Indeed, we and others have shown that EBNA-3, EBNA-4, and EBNA-6 form stable complexes with the cellular DNA-binding factor RBP-Jκ/2N, leading to inhibition of EBNA-2-mediated modulation of gene expression (21, 24, 30, 45). Therefore, the EBNA 3 gene family may function by differentially regulating viral and cellular genes whose promoters contain RBP-Jκ/2N-binding sites (31, 42), illustrating the importance of maintaining adequate control over the expression levels of EBNA-3, EBNA-4, and EBNA-6.

The three genes of the EBNA 3 family have a similar genomic organization, consisting of two exons separated by a small intron (Fig. 1). These genes encode nuclear proteins of 944, 938, and 992 amino acids (aa), respectively, and have structural and sequence homologies (22, 33). The two types of EBV are divergent, at the nucleotide level, within the coding regions of the EBNA 3 family of genes (33). In contrast, the intronic regions of these genes are remarkably homologous (95 to 98%) between A- and B-type viruses, indicating a selective conservation despite the evolutionary emergence of two distinctive EBV types. In LCLs, the EBNA genes are transcribed into a ≈110-kb primary multicistronic transcript which originates from either the Cp or Wp promoters (44). The Cp promoter itself can be activated by the EBNA-2 protein via RBP-Jκ/2N, thereby providing a mechanism for control of viral gene expression at the transcriptional level (32). However, only limited data is available on the metamorphosis of the primary transcript into the fully processed EBNA-3, EBNA-4, and EBNA-6 mRNAs. Proposed posttranscriptional mechanisms include alternative splicing and polyadenylation site selection, resulting in a very low abundance of mRNAs containing the EBNA 3 family genes (38).

There is no evidence that expression of the EBNA-3,

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EBNA-4, and EBNA-6 genes can be individually regulated. However, the strong conservation of the introns of the EBNA 3 gene family prompted us to investigate their potential in regulating gene expression. Indeed, this study demonstrates that the vast majority of mRNA containing EBNA-3, EBNA-4, and EBNA-6 contain intronic sequences and provides evidence that EBV may use this intron retention to fine-tune the expression of the EBNA 3 family genes.

MATERIALS AND METHODS

Cell lines. QIMR-ISM (Wil), QIMR-MP (B95.8), QIMR-SB (B95.8), and QIMR-KT (B95.8) are EBV-positive LCLs established by infection of human B lymphocytes from donors ISM, MP, SB, and KT with the EBV A-type virus strains Wil or B95.8. MutuIII-c95 is an EBV-positive group III BL cell line (13). All of these cell lines expressed the full set of EBV latent antigens as indicated by immunoblot and cytotoxic T-cell assays. DG75 is an EBV-negative BL cell line (4). Cells were maintained in RPMI 1640 medium supplemented with 2 mM glutamine, 60 μg of benzylpenicillin per ml, 100 μg of streptomycin per ml, and 10% fetal calf serum (growth medium) at 37°C in a 5% CO_2 atmosphere.

Expression vector constructs. The generation of EE3, EE4, EE6, EE346, and vector control transfectants has been reported elsewhere (19). Briefly, the episome-based shuttle vector EBO-pLPP (23), which contains the oriP/EBNA-1 replicon of EBV, a simian virus 40 (SV40) transcriptional cassette, and the hygromycin resistance gene, was used to generate expression vectors containing the genes of the EBNA 3 family. The EBNA-3, EBNA-4, and EBNA-6 genes were derived from plasmid pACYC184-H3E, which contained the genomic *Hin*dIIIE fragment of A-type EBV strain M-ABA (27).

To generate expression vectors encoding mutated forms of EBNA-3, the *Hin*dIII-*Cel*II fragment of plasmid pACYC184-H3E (positions 91821 to 95369 according to the B95.8 sequence [3] containing the genomic EBNA-3 gene sequence) was subcloned into pBluescriptKS (Stratagene) (*Hin*dIII-*Xba*I end filled). This vector construct was used to modify EBNA-3 or cut it with the appropriate enzymes shown in Fig. 6A, and the mutated EBNA-3 sequences were then shuttled back into the expression vector EBO-pLPP by using *Hin*dIII and *Not*I. A frameshift mutation (EBO-E3M) was introduced into exon 1 of EBNA-3 by digestion with *Bgl*II (position 92538) (3), end filling with T4 DNA polymerase and religation. To generate a FLAG-tagged EBNA-3, an oligo-adapter containing a *Bam*HI-*Bgl*II-flanked FLAG epitope sequence (DYKD-DDK) was inserted in frame into the *Bgl*II site of exon 1 of EBNA-3. The expression plasmid EBO-EBNA3(S), which contained the spliced form of EBNA-3, was created by exchanging the genomic *Bgl*II-*Bgl*II fragment of EBNA-3 (which contained the end of exon 1, the intron, and part of exon 2 [positions 92538 to 93424 according to the B95.8 sequence]) with the *Bgl*II-*Bgl*II fragment of the cDNA of EBNA-3. The genomic *Hin*dIII-*Cel*II fragment containing the EBNA-3 gene was also subcloned into the episomal expression vector pREP4 (Invitrogen) by using *Hin*dIII and *Not*I, thereby creating plasmid REP-E3. In this plasmid, EBNA-3 transcription was driven by the Rous sarcoma virus (RSV) promoter. To generate an EBNA-1-deleted EBNA-3 expression vector (REP/DE1-E3), pREP4 was cut with *Sac*I and *Stu*I (thereby deleting the N-terminal 486 aa of the 641-aa EBNA-1 coding region), end filled, and religated, and EBNA-3 was cloned into its *Hin*dIII and *Not*I polylinker sites.

Cell transfection. Exponentially growing DG75 cells (3×10^6 to 5×10^6) were washed in growth medium, transfected in growth medium with plasmid DNA (12 μg for stable transfection; up to 25 μg for transient transfection) with the Bio-Rad Gene Pulser (960 μF, 240 V, 0.4-cm gap electrode, room temperature, 350-μl assay volume), and finally resuspended in 5 ml of growth medium. After 2 days, the cells were either analyzed for transient expression or selected with 600 μg of hygromycin B (Boehringer Mannheim, Castle Hill Australia) per ml. Polyclonal transfectants grew out after 2 to 3 weeks and were stably maintained in hygromycin B-containing growth medium. Parental DG75 cells were stably transfected with the individual EBO-EBNA3, EBO-EBNA4, and EBO-EBNA6 expression vectors (EE3, EE4, and EE6), with vector EBO-EBNA346 encoding the complete EBNA 3 gene family (EE346), and with constructs expressing mutated or truncated forms of EBNA-3 or with the control vector EBO-pLPP alone.

RNA purification. Total cellular RNA and mRNA was prepared from 5×10^6 to 10×10^6 exponentially growing cells by using one of three commercially available RNA kits which were based on the guanidinium thiocyanate purification method (RNAgents Total RNA isolation system, Promega; Total RNA isolation reagent, Advanced Biotechnologies; QuickPrep Micro mRNA purification kit, Pharmacia). Total RNA (40 μg) or mRNA (4 μg) was incubated with 30 U of RNase-free DNase I (Boehringer Mannheim) in the presence of PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$), 40 U of RNase inhibitor (Boehringer Mannheim), and 5 mM dithiothreitol for 50 min at 37°C in a total volume of 80 μl. The RNA was finally purified by phenol-chloroform extraction followed by isopropanol precipitation. The purity of the RNA was determined by measuring the absorbance at 260 and 280 nm ($A_{260/280}$), and its integrity was verified on a formamide-agarose gel. Alternatively, the DNase I treatment of RNA was performed before the addition of both the reverse transcriptase (RT) enzyme and oligo(dT) primers in RT buffer. The DNase I was heat inactivated (5 min at 90°C followed by 5 min at 70°C) and the samples were directly used for the RT reaction.

RNA fraction and polysome gradient. Fractionation of RNA was performed by modified protocols (8, 16). Cells (4×10^7) were washed twice in ice-cold phosphate-buffered saline (PBS) and were then disrupted with 0.5 ml of a modified Nonidet P-40 (NP-40) lysis buffer (0.5% NP-40, 10 mM HEPES [pH 7.6], 10 mM NaCl, 3 mM CaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 100 U of RNasin per ml) for 1 min on ice. After high-speed centrifugation (2 min at 4°C), the cytoplasmic supernatant was collected and the cytoplasmic RNA was either extracted by using the Total RNA isolation reagent kit or applied to a sucrose gradient (10 to 50%, wt/vol). A step sucrose gradient was prepared by overlaying 0.75 ml of 50, 40, 30, 20, and 10% sucrose-containing buffer (10 mM HEPES [pH 7.6], 10 mM NaCl, 3 mM CaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 50 U of RNasin per ml), and a linear gradient was formed by diffusion at 4°C for 16 h. The cytoplasmic fraction was split in half, one sample was adjusted to 40 mM EDTA, and both samples were layered on a sucrose gradient with or without EDTA (40 mM), respectively. The gradients were centrifuged at 55,000 rpm for 75 min at 4°C in a Beckman SW60Ti rotor. Ten fractions (0.4 ml) were collected by bottom puncture of the tube, and the A_{260} of the fractions was measured. The RNA was purified with the Total RNA isolation reagent kit and finally resuspended in 15 μl of RNase-free water.

RT-PCR and DNA-PCR analysis. A detailed description of the RT assay was described recently (20). Briefly, purified total RNA (1 μg), mRNA (200 ng), or gradient-fractionated RNA (5 μl per fraction sample) was reverse transcribed at 42°C in the presence of oligo(dT) primers, deoxynucleoside triphosphates, and SuperScript-II enzyme as specified by the manufacturer (GibcoBRL, Melbourne, Australia) in a 20-μl assay mixture. For RT-negative controls, the SuperScript-II enzyme was omitted. Strand-specific RT reactions were performed under high-stringency conditions at 56°C with 2 pmol of a sequence-specific primer and SuperScript-II enzyme.

PCR amplifications (20 μl) were performed in PCR buffer containing 0.5 μM each primer, 1 to 2 μl of first-strand cDNA sample or 10 to 50 ng of DNA, 200 μM each deoxynucleoside triphosphate, and 1 U of AmpliTaq DNA polymerase (Perkin-Elmer). The ice-chilled samples were transfered to a 9600 GeneAmp PCR instrument system (Perkin-Elmer), preheated at 85°C, and subjected to an initial denaturation at 94°C for 1 min. This was followed by 30 to 35 cycles of denaturation at 94°C for 20 s, primer annealing at 56 to 58°C for 30 s, and primer extension at 72°C for 30 s and then a final extension at 72°C for 5 min. The amplified products were separated by electrophoresis on 2.5 to 3% agarose gels containing ethidium bromide in TAE buffer (40 mM Tris acetate, 1 mM EDTA [pH 8.0]). The gel was photographed under UV light with Polaroid T-55 film, and the relative amount of each DNA band was quantified using a Computing Densitometer 300B system (Molecular Dynamics, Sunnyvale, Calif.).

Primers. The following oligonucleotide primers were used for amplification of EBNA-3, EBNA-4, EBNA-6 (Fig. 1), and LMP-1 (their positions within the B95.8 sequence [3] are given in parentheses): E3-intron-F, 5′-CTAAGAACA CTTCTTCAAGC (92547); E3-intron-R, 5′-CTCGGTATTTGAAATCTGG (92726); E3-intron-R2, 5′-GATCCGAAAAACTGGTCTA (92707); E3-intron/internal-F, 5′-GTGAGCATCCCTATGGC (92582); E3-intron/internal-R, 5′-C TGAAACCAACGGCAACA (92669); E3-YPL-F, 5′-GACGAGACAGCTA CCAG (93624); E3-YPL-R, 5′-GAGATACAGGGGGCAAG (93780); E4-intron-F, 5′-GGGATCTGAGCCTATTTCAC (95457); E4-intron-R, 5′-TTC CAACGCCTCTGCTTAAC (95933); E6-intron-F, 5′-GACATCACACCATAT ACCGC (98674); E6-intron-R, 5′-TGTTAGAAGCCAATGTCGCC (98966); LMP1-intron-F, 5′-GATGAGGTCTAGGAAGAAGG (168884); and LMP1-intron-R, 5′-TCGCTCTCTGGAATTTGCAC (169113). The cDNA fragments of β_2-microglobulin (β_2M, 131 bp), β-actin (410 bp), or EBNA-1 (240 bp) were amplified with primers β_2M5′ (5′-CCCCCACTGAAAAAGATGAG) and β_2M3′ (5′-TCACTCAATCCAAATGCGGC), p5′Ac and p3′Ac (41), or E1-4-F (TTACAACCTAAGGCGAGGAA) and E1-6-R (ACAGTCACCCTG ATATTGCA), respectively.

Northern blot analysis. Radiolabelled DNA probes were generated by PCR amplification with the genomic EBNA-3 sequence and either primers E3-intron/internal-F and E3-intron/internal-R (intron) or E3-YPL-F and E3-YPL-R (exon 2) (Fig. 1). A radiolabelled probe of β-actin was generated from oligo(dT) reverse-transcribed cDNA of DG75 cells by using the β-actin-specific primers p5′Ac and p3′Ac. The PCRs (100-μl mixtures) were performed for 30 cycles as described above, except that 50 μCi of each [α-^{32}P]dATP and [α-^{32}P]dCTP (3,000 Ci/mmol; DuPont) were added and 200 μM dGTP and dTTP and 6.3 μM dATP and dCTP were used. The same PCR conditions were used to generate radiolabelled single-stranded antisense EBNA-3 probes, except that the 5′ primer was used at a 50-fold-lower concentration (25).

mRNA (4 μg) was electrophoresed on a 1% agarose–formaldehyde gel in MOPS/EDTA buffer (20 mM MOPS [3-(*N*-morpholino)propanesulfonic acid], 5 mM sodium acetate, 1 mM EDTA [pH 7.0]) at 55 V (constant voltage). After electrophoresis, the RNA was transfered to a Hybond-N membrane (Amersham) by capillary blotting and fixed onto the membrane by heating at 80°C for 2 h under vacuum. The membranes were incubated for 1 h in hybridization buffer (50% formamide, 5× SSPE [1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA, pH 7.7], 5× Denhardt's solution, 0.5% sodium dodecyl sulfate [SDS], 20 μg of salmon sperm DNA per ml), and radiolabelled probe was added and hybridized at 42°C overnight. The filter was washed twice in 2× SSPE–0.1%

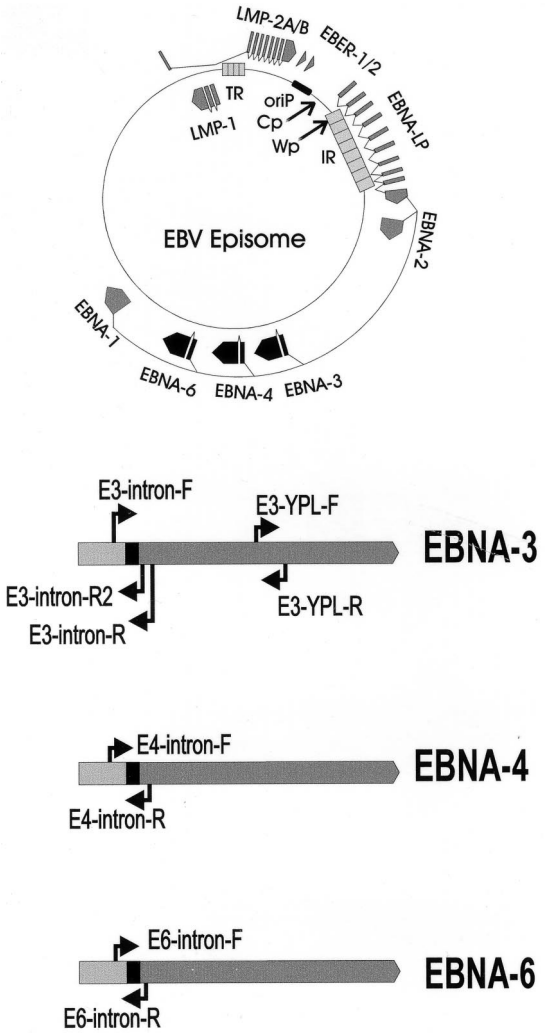

FIG. 1. Schematic diagram of EBV genes. The genomic organization of the EBV genome and the two promoters driving EBNA gene expression is depicted. The diagram is not drawn to scale. The gene structure of the EBNA 3 family is illustrated, and the names and positions of primers are indicated. Introns and exons are indicated in black and grey, respectively.

SDS at room temperature for 10 min and once in 1× SSPE–0.1% SDS at 42°C for 15 min and finally subjected to a high-stringency wash in 0.1× SSPE–0.1% SDS at 42°C for 10 min. The blot was exposed to a Kodak Storage Phosphor Screen at room temperature for 3 to 4 days for the EBNA-3 probes and for 1 day for the β-actin probe, and the signals were analyzed on a PhosphorImager 400B system (Molecular Dynamics). The filter was stripped by being washed three times in boiling 0.1% SDS for 30 min each prior to reprobing.

S1 nuclease protection assay. To generate the S1 probe, the *Hin*dIII-*Bam*HI fragment of EBNA-3 (positions 91821 to 92705 [3] containing exon 1, the intron, and the start of exon 2 [see Fig. 6A]) was cloned into pBluescriptKS with the same enzyme sites and then linearized with *Bgl*II (which cut in exon 1). A radiolabelled single-stranded DNA which was complementary to the EBNA-3 coding strand was generated by PCR in an 80-μl reaction mixture containing PCR buffer, 200 ng of plasmid DNA, 312 nM M13 forward primer (which bound to the plasmid backbone), 62.5 μM each dATP, dGTP, and dTTP, 1.25 μM dCTP, 10 μl of [α-^{32}P]dCTP (10 mCi/ml; 3,000 Ci/mmol [DuPont]), and 2.5 U of *Taq* polymerase with 30 cycles of denaturation at 95°C for 60 s, primer annealing at 52°C for 60 s, and primer extension at 72°C for 90 s. The PCR products were ethanol precipitated, and the single-stranded S1 probe was purified on a 10% polyacrylamide–8 M urea gel. The sequence composition of this 265-nucleotide large probe is depicted in Fig. 5.

The S1 nuclease protection assay was performed with the S1 assay kit (Ambion, Austin, Tex.) as recommended by the manufacturer. Briefly, the radiolabelled S1 probe was hybridized with mRNA or total RNA of cells expressing EBNA-3. For controls, unrelated RNA from yeast or in vitro-transcribed RNA from genomic or cDNA forms of EBNA-3 (both of which were cloned into pBluescript and induced with the T3 promoter) were used. The single-stranded, unprotected molecules were digested with S1 nuclease, and the remaining products were separated on a denaturing 10% polyacrylamide gel. The dried gels were exposed to X-ray film overnight.

Immunoblot analysis. Cells were washed in PBS and lysed by being placed in sample buffer (2% SDS, 5% α-monothioglycerol, 10% glycerol, 60 mM Tris-HCl [pH 6.8], 0.001% bromophenol blue) with sonication and then boiled for 5 min. Samples were subjected to SDS-polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose filters (Amersham) with a minigel system (Bio-Rad). The filters were stained with Ponceau S (Sigma) to confirm the presence of even amounts of protein and were then preincubated for 1.5 h in Blotto buffer (PBS, 0.1% Tween 20, 5% skim milk, 1% bovine serum albumin). For detection of the EBNA-3 and EBNA-1 proteins, the filters were incubated with the EBV-positive human serum MCr (diluted 1:50 in Blotto) for 1 h at room temperature. For detection of the FLAG epitope EBNA-3, the commercial mouse anti-FLAG M2 antibody (Kodak) was used. The filters were washed four times for 10 min each in PBS–0.1% Tween 20 and incubated for 1 h with either horseradish peroxidase-conjugated sheep anti-human immunoglobulin G (Amersham) or horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin G (Silenus) at a 1:1000 dilution. The filters were washed as outlined above, and the reactions were visualized by using the enhanced chemiluminescence Western blotting detection system (Amersham). The relative amount of each protein band was quantified with a Computing Densitometer 300B system (Molecular Dynamics).

RESULTS

Intronic sequences are maintained in mRNA for EBNA-3, EBNA-4, and EBNA-6. Vectors containing the genomic sequences of EBNA-3, EBNA-4, and EBNA-6 were stably transfected into DG75 cells. The expression vector (EBO-pLLP) contained the EBV-encoded EBNA1/oriP replicon enabling episomal replication of the plasmid and an SV40 transcriptional cassette driving recombinant gene expression. Cell clones and bulk cultures of transfected cells containing the complete gene family of EBNA 3 were designated EE346, whereas EE3, EE4, and EE6 cells expressed the EBNA-3, EBNA-4, and EBNA-6 proteins, respectively. Control cells (E) were transfected with the expression vector only.

Total RNA and mRNA was prepared from transfected cells and subjected to DNase I treatment, which we have previously shown to be essential for the RT-PCR amplification of episome-based genes (20). By using oligo(dT) primers, the polyadenylated RNA was reverse transcribed and the resulting first-strand cDNA was PCR amplified with primer pairs flanking the introns of EBNA-3, EBNA-4, and EBNA-6 (Fig. 1). Two cDNA species were obtained and were similar in size to the spliced and unspliced control PCR products amplified from DNA sequences of the genomic or cDNA versions of the EBNA genes (Fig. 2A to C). Sequencing of these RT-PCR products confirmed that they were indeed derived from spliced and unspliced cDNA. The absence of any PCR products in the RT-negative controls (Fig. 2, lanes −) ruled out any detectable contamination by genomic viral sequences. Several repetitions of the experiment with transfected bulk cultures or cell clones gave the same results and suggested that a significant proportion of the EBNA-3, EBNA-4, and EBNA-6 mRNA transcripts retained intron sequences.

The possibility that the RT-PCR products resulted from RNA transcripts, expressed from the negative DNA strand, was addressed by generating strand-specific cDNA. Strand-specific RT reactions for the EBNA-3 intron were performed under high-stringency conditions with either primer E3-intron-F or E3-intron-R (Fig. 1), with total RNA or mRNA prepared from EE3 and EE346 transfectants. The first-strand cDNA samples were then PCR amplified with the same primers. PCR amplification of cDNA generated by the 3′ primer (E3-intron-R) resulted in the production of both spliced and unspliced products, again with a predominance of the unspliced form (Fig. 2D, lanes 3′). No products were detected

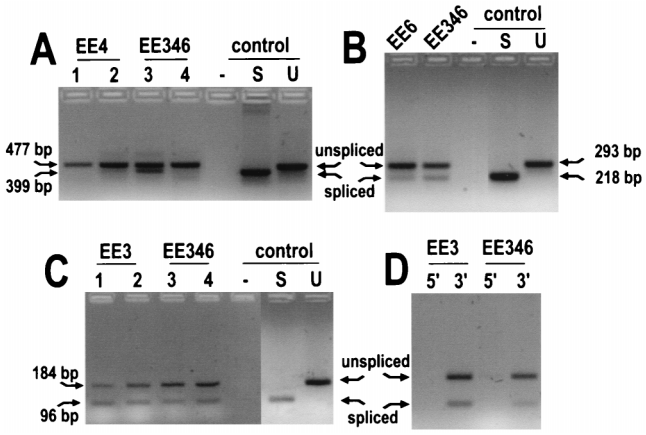

FIG. 2. Intron detection by RT-PCR from stably transfected cell lines. (A and C) mRNA (lanes 1 and 3) and total RNA (lanes 2 and 4) were prepared from DG75 cells expressing the complete family of EBNA 3 genes (EE346) or the individual EBNA-3 (EE3) and EBNA-4 (EE4) genes (A and C). (B) Total RNA of EE6 and EE346 cells was prepared. The RNA was reverse transcribed with oligo(dT) primer, and the RT was omitted in an RT-negative control for the EE346 RNA (−). The intron regions of EBNA-4 (A), EBNA-6 (B) and EBNA-3 (C) were PCR amplified from the first-strand cDNAs or from plasmid DNA containing the genomic (U) or cDNA (S) sequence of the EBNA-4, EBNA-6, or EBNA-3 genes. (D) mRNA from EE3 and total RNA from EE346 cells were prepared. Strand-specific RNA was reverse transcribed with either sense primer E3-intron-F (5′) or antisense primer E3-intron-R (3′), and the first-strand cDNAs were PCR amplified with both primers. All of the PCR products were separated on a 2.5% agarose gel and visualized by ethidium bromide staining. The positions and lengths (in base pairs) of the unspliced and spliced products are indicated.

when the 5′ primer (E3-intron-F)-generated cDNA sample was PCR amplified (Fig. 2D, lanes 5′). These results demonstrated that the unspliced EBNA-3 transcript did not originate from an antisense RNA transcript and confirmed that the results were not due to contamination with genomic EBNA-3 sequences.

Intron retention is specific for the EBNA 3 family genes. To determine whether intron retention also occurred in EBV-infected cell lines, RT-PCR was performed on total RNA and mRNA isolated from a number of different LCLs (QIMR-ISM, QIMR-KT, QIMR-NB, QIMR-PGP, QIMR-SB, and B95.8) as well as the BL-cell line MutuIII-c95. The results show that the vast majority of EBNA-3, EBNA-4, and EBNA-6 mRNA were unspliced in each of the EBV-positive cell lines (Fig. 3A to C). The absence of any PCR-generated product in the RT-negative controls (Fig. 3, lanes −) confirmed that there was no genomic DNA contamination.

To determine whether retention of intron sequences within mRNA transcripts was restricted to the EBNA 3 family genes, processing of transcripts from the latent viral LMP-1 gene (which contains two small introns with nonconsensus splice acceptor and donor sequences) was also analyzed by RT-PCR. Primers LMP1-intron-F and LMP1-intron-R, which flanked the second intron of LMP-1, were used to PCR amplify the first-strand cDNAs generated from total RNA and mRNA from different EBV-positive cell lines. As shown in Fig. 3D, the spliced LMP-1 cDNA was the dominant form in both LCLs and the BL-cell clones. These results indicate that intron retention is specific for the genes of the EBNA-3 family.

Northern blot and S1 nuclease protection assays confirm that EBNA-3 mRNA contains intronic sequences. To verify the RT-PCR data, Northern blot and S1 nuclease protection assays were used to analyze the processing of the EBNA-3 RNA. mRNA preparations from EE3 and vector control transfectants, as well as from EBV-infected cells, were analyzed by Northern blotting. The β-actin control demonstrated the integrity of the RNA and showed that similar amounts of the RNA were present in each sample (Fig. 4, β-actin). A radiolabelled EBNA-3 intron probe was generated by PCR amplification with primers E3-intron/internal-F and E3-intron/internal-R, which were specific for the intron sequence only. The intron probe hybridized with a ≈4.7-kb mRNA (calculated by comparison to the rRNAs) present in both LCLs and in EE3 cells but not in the vector control transfectants (Fig. 4, intron). Hybridization with a higher-molecular-weight mRNA also occurred in the sample derived from the LCLs (Fig. 4, intron, lane 3). The use of a radiolabelled, single-stranded antisense intron probe, generated by asymmetric PCR, and mRNA prepared from three different LCLs gave the same results (data not shown). The size of the intron-containing EBNA-3 mRNA correlated well with previously published data showing that EBNA-3 is encoded by a 4.5- to 5-kb mRNA (15, 34).

To determine the percentage of intron-containing EBNA-3 mRNA compared to fully spliced EBNA-3 mRNA, the filter was deprobed and rehybridized with a radiolabelled probe specific for the second exon of EBNA-3. This probe was generated by PCR amplification with primers E3-YPL-F and E3-YPL-R (Fig. 1). The exon probe, which detects both unspliced and spliced transcripts, resulted in the same hybridization pattern and similar signal strength with respect to the intron probe (Fig. 4, exon). The results indicate that the majority of the EBNA-3 4.7-kb mRNA (as detected by the exon probe) contained intron sequence (as detected by the intron probe). The additional larger mRNA species, present in LCLs, to which the intron and exon probes of EBNA-3 hybridized was likely to consist of partially processed primary transcripts. When total RNA of EE3 cells or LCLs was used as a target, no hybridization signals could be detected with either the intron or exon probes (data not shown), consistent with the low abundance of EBNA-3 mRNA (18).

An S1 nuclease protection assay was performed to quantify the ratio of unspliced to spliced RNA within the same sample. A radiolabelled single-stranded antisense DNA probe which covered the EBNA-3 intron and its flanking exon regions was prepared. The probe also carried a random sequence of 98 nucleotides at its 5′ tail. The expected digestion patterns of the probes are illustrated in Fig. 5. For controls (Fig. 5, lanes 3 and 4), RNA was transcribed in vitro from the genomic or cDNA form of EBNA-3 and the purified RNA and the probe were subjected to the S1 nuclease assay. The intron-containing control RNA protected the EBNA-3 sequence within the probe (167 n) but not its nonhomologous tail. The in vitro-transcribed RNA of the EBNA-3 cDNA gave rise to the expected fragments (40 n and 39 n). Surprisingly, a higher-molecular-weight fragment, which may have been the result of stable intermediate structures within the intronic sequence of the EBNA-3 RNA, was also present. However, the control reaction with nonhomologous yeast RNA in the presence or absence of S1 nuclease gave the expected results, indicating optimal S1 treatment conditions (Fig. 5, lanes 5 and 6). To measure the degree of EBNA-3 intron retention in EBV-infected or -transfected EE346 cells, the mRNA of the LCL QIMR-MP or total RNA of EE346 cells was subjected to the S1 nuclease assay. The protected unspliced probe fragment was prominent, in contrast to the bands representing the spliced EBNA-3 RNA (Fig. 5, lanes 1 and 2), indicating that very little spliced RNA was present. Treatment of the RNA with DNase I prior to the S1 nuclease assay did not abolish the presence of the unspliced probe fragment. In contrast, pretreatment of the RNA with RNase A resulted in the complete

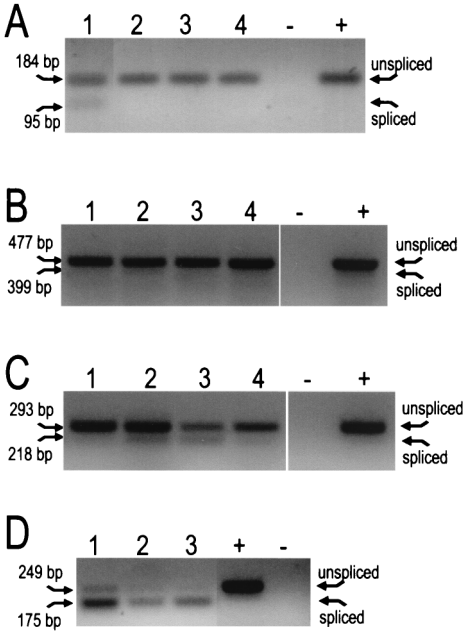

FIG. 3. RT-PCR detection of introns from EBV-infected cell lines. Total RNA of MutuIII-c95 (lane 1), QIMR-ISM (lane 2), and QIMR-KT (lane 4) and mRNA from QIMR-KT cells (lane 3) were reverse transcribed with an oligo(dT) primer. The RT was omitted in a control for the QIMR-KT mRNA (−). The intron regions of EBNA-3 (A), EBNA-4 (B), EBNA-6 (C), and LMP-1 (D) were PCR amplified from the first-strand cDNAs or from genomic DNA of QIMR-KT cells (+). The PCR products were separated on a 2.5% agarose gel and visualized by ethidium bromide staining. The positions and lengths (in base pairs) of the unspliced and spliced products are indicated.

digestion of the unprotected probe (data not shown). This data confirmed the RT-PCR and Northern blot results, demonstrating that the vast majority of the EBNA-3 mRNA contained intronic sequence.

Analysis of elements possibly involved in EBNA-3 intron retention. Proteins expressed by alternatively spliced genes (e.g., sxl of *Drosophila*) can autoregulate their own splicing (36). To address whether the EBNA-3 protein itself may have been involved in preventing the splicing of its own transcript, a frameshift was introduced into the genomic EBNA-3 sequence (EBO-E3M; Fig. 6A). This mutated EBNA-3 gene was stably expressed in DG75 cells. The frameshift, introduced into the first exon of EBNA-3, allowed transcription of mRNA but prevented translation of the EBNA-3 protein. Sequencing of the mutated EBNA-3 gene verified the frameshift, and immunoblot analysis of the transfectants confirmed that the EBNA-3 protein was not expressed (data not shown). RT-PCR analysis of mRNA preparations of EBO-E3M cell transfectants showed that a high proportion of the mutated EBNA-3 mRNA still contained the intron (Fig. 6B, lane EBO-E3M). This result was confirmed by Northern blot analysis (data not shown) and indicated that expression of the EBNA-3 protein was not necessary for retention of intronic sequence in the EBNA-3 mRNA.

To determine whether a region of the EBNA-3 mRNA contained elements involved in intron retention, a series of deletion mutants were prepared, cloned into an expression vector (EBO-pLPP), and stably expressed in DG75 cells (Fig. 6A). The mRNA preparations from these transfectants were analyzed by RT-PCR. Deletion of the 3′ untranslated region and most of the second exon of the EBNA-3 mRNA alleviated intron retention and resulted in predominantly spliced forms of the EBNA-3 mRNA (EBO-E3BamHI; Fig. 6B). This suggested that the mRNA downstream of the *Bam*HI site may have contained an element involved in intron retention. Consequently, overlapping regions of the second exon were cloned behind the *Bam*HI site to try to identify this element (EBO-E3HpaI, EBO-E3BseRI, EBO-E3EcoNI, and EBO-E3StuI; Fig. 6A). Addition of any sequence 3′ of the *Bam*HI site resulted in retention of the intron (Fig. 6B). One region in common in all of these constructs was the EBV-encoded EBNA-3 polyadenylation signal. To address the possible involvement of this element, two constructs were created, one containing the natural poly(A) signal (EBO-E3AATAAA) and the other containing a mutated poly(A) signal (EBO-E3TTTAA) (Fig. 6A). RT-PCR was again used to determine the degree of splicing of the mRNA expressed by these constructs; however, both resulted in intron retention (Fig. 6B). Finally, the 3′ untranslated region of EBNA-3 was completely removed, but again RT-PCR showed the presence of intronic sequences (EBO-E3KpnI; Fig. 6). Although Fig. 6B shows a slight decrease in intron retention in the constructs of EBO-E3TTTAA and EBO-E3StuI, the results presented at the right-hand side of Fig. 6A were a summary of at least three different experiments showing that the only construct consistently spliced was EBO-E3BamHI. These results suggested that there was no element, within the EBNA-3 mRNA, that was directly involved in intron retention but, rather, that the spatial conformation of the RNA seemed to be involved in the incomplete splicing of EBNA-3.

The transcriptional cassette of the expression vector used contained the SV40 late polyadenylation signal, which was located behind the EBV-encoded EBNA-3 poly(A) signal. Since alternative poly(A) site selection can affect splicing (11), we determined whether a preferential use of the EBV or SV40 poly(A) sites might be linked to intron retention. The truncated forms of EBNA-3 mRNA, which either were predominantly spliced (EBO-E3BamHI) or retained the intronic sequence (EBO-E3StuI, EBO-E3TTTAAA), were amplified by RT-PCR and sequenced to determine the poly(A) signal used (data not shown). If both the EBV- and the SV40-encoded polyadenylation signals were present together in the construct (EBO-E3StuI), the unspliced as well as the spliced mRNA of EBNA-3 utilized the EBV EBNA-3 poly(A) signal. Where the EBV poly(A) signal was either absent (EBO-E3BamHI) or mutated (EBO-E3TTTAAA), both spliced and unspliced EBNA-3 mRNA contained the SV40 poly(A) signal, indicating

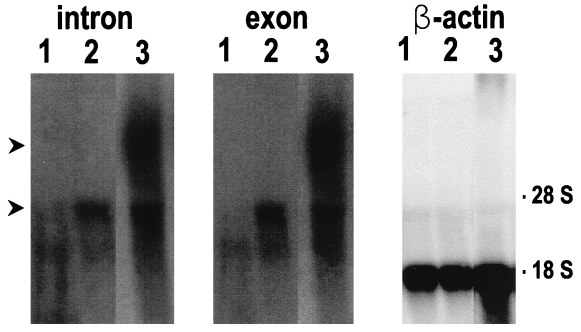

FIG. 4. EBNA-3 intron detection by Northern blotting. mRNA was prepared from transfected DG75 control cells (lane 1), EE3 cells (lane 2), or EBV-positive QIMR-ISM cells (lane 3). The mRNA was electrophoresed on formaldehyde gels, transferred to nitrocellulose, and hybridized with radiolabelled probes from the EBNA-3 intron (intron), the second EBNA-3 exon (exon), or β-actin. The positions of rRNA are indicated. Hybridization to EBNA-3 RNA is indicated by arrowheads. Note that lanes 1 and 2 were derived from one autoradiograph and lane 3 was derived from another.

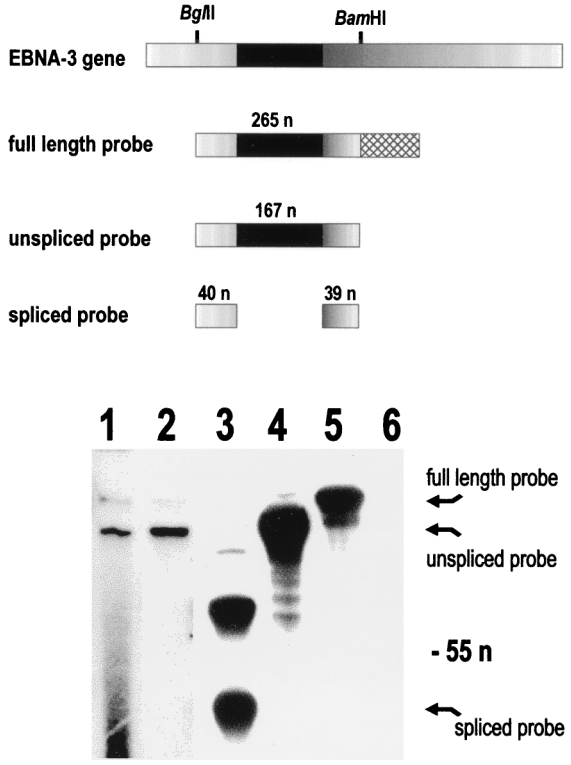

FIG. 5. EBNA-3 intron detection by S1 nuclease protection assay. (Top) Schematic diagram of the EBNA-3 gene with the restriction sites used for generating the probe and the nucleotide length (n) of the probe and its protected fragments. First and second exons are shaded in grey, the intron is black, and the nonhomologous 5′ tail of the probe is cross-hatched. (Bottom) mRNA of QIMR-MP cells (lane 1) and total RNA of EE346 cells (lane 2) were prepared. For controls, either the cDNA (lane 3) or the genomic (lane 4) form of EBNA-3 RNA was transcribed in vitro or total RNA of yeast was used (lanes 5 and 6). The RNA was hybridized with a radiolabelled antisense EBNA-3 probe carrying a nonhomologous 5′ tail, and the samples were treated with S1 nuclease (lanes 1 to 5) or left untreated (lane 6). The products were separated on a 10% denaturing sequencing gel and visualized by autoradiography. The positions of the probe fragments and the xylene blue marker (55 n) are indicated.

that there was no link between EBNA-3 intron retention and the polyadenylation signal used.

The potential involvement of either the SV40 promoter (driving EBNA-3 transcription) or EBNA-1 (necessary for episomal replication) in EBNA-3 intron retention was also analyzed (Fig. 6A). The EBNA-3 gene was cloned between the RSV promoter and the SV40 poly(A) site of the episomal expression vector pREP4, creating expression vector REP-E3 (Fig. 6A). This plasmid was either transiently or stably transfected into DG75 cells, and immunoblot analysis of the cell extracts confirmed expression of the EBNA-3 protein (data not shown). RT-PCR analysis of RNA prepared from the transfected cells showed a predominance of EBNA-3 mRNA containing intronic sequence (Fig. 6B, REP-E3). Finally, an EBNA-3 expression vector, which was based on REP-E3 but deleted in EBNA-1, was constructed (Fig. 6A, REP/DE1-E3) and transiently transfected into DG75 cells. Again, RT-PCR analysis showed a predominance of intron-containing EBNA-3 mRNA in the RNA from these cells (Fig. 6B, REP/DE1-E3), indicating that EBNA-1 was not involved in maintaining intronic sequence in the EBNA-3 mRNA.

Unspliced mRNA of the EBNA 3 family genes is transported into the cytoplasm and associates with polysomes. Retention of the introns within the EBNA 3 family mRNAs could regulate protein expression by preventing the transport of mRNA into the cytoplasm. To address this, an EBV-positive LCL (QIMR-SB) was separated into nuclear and cytoplasmic fractions by using an NP-40 based lysis buffer. The cytoplasmic RNA fraction was extracted and analyzed by RT-PCR with oligo(dT) and primers flanking the intron of each of the genes of the EBNA 3 family (Fig. 1). For controls, a 131-bp cDNA fragment of the cellular β_2-microglobulin gene was also amplified. As demonstrated in Fig. 7A, the EBNA-3, EBNA-4, and EBNA-6 mRNAs present in the cytoplasmic fraction were predominantly unspliced (only EBNA-3 showed some detectable spliced forms). This data indicated that the EBNA 3 family mRNAs were transported into the cytoplasm, even though they still contained introns, and raised the question whether these mRNAs were free in the cytoplasm or associated with polysomes.

Therefore, polysomes were prepared by sucrose gradient centrifugation of cytoplasmic extracts from the LCL QIMR-SB. These were analyzed by RT-PCR to determine if the

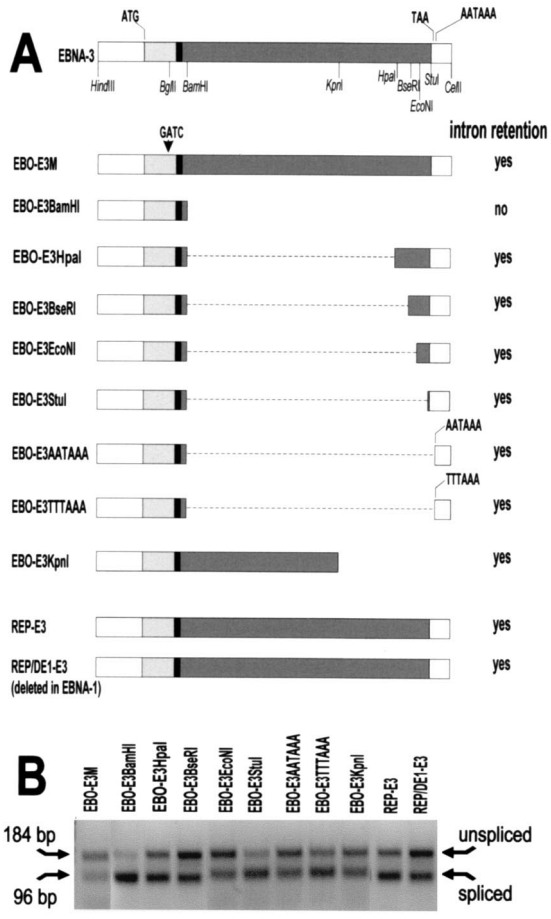

FIG. 6. EBNA-3 intron retention in deletion constructs. (A) Schematic diagram of EBNA-3 mutants showing the nucleotide insertion GATC (arrow) and restriction sites used for cloning. The expression vector used is represented in the initials of the constructs, EBO (EBO-pLPP) or REP (pREP4). REP/E1-E3 is deleted in EBNA-1. Translation and polyadenylation signals, as well as the nontranslated regions (white), the exons (grey), and the intron (black) of EBNA-3, are illustrated. The level of intron retention (summarized from at least three different experiment) is given on the right. (B) RT-PCR with EBNA-3 intron-flanking primers (E3-intron-F and E3-intron-R2) and mRNA from stable transfectants expressing EBNA-3 deletion constructs. Transient transfectants of the REP-E3 and REP/DE1-E3 plasmid transfectants were analyzed. For RT-PCR details, see the legend to Fig. 3.

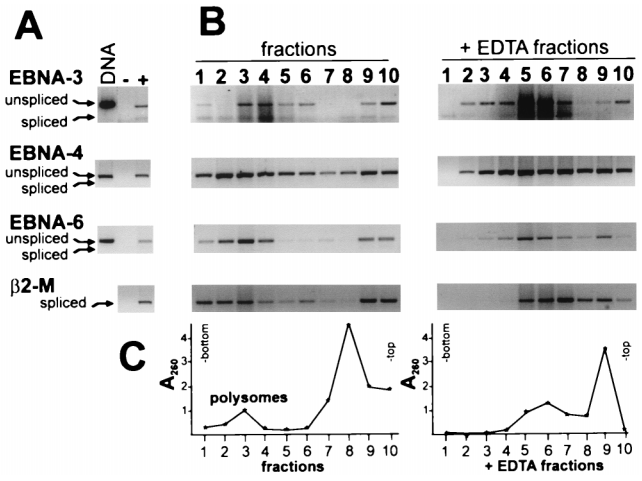

FIG. 7. RT-PCR of fractionated cytoplasmic RNA. (A) RNA was prepared from the cytoplasm of the LCL QIMR-SB and reversed transcribed with an oligo(dT) primer (+). As a control, RT was omitted (−). The spliced and unspliced cDNA fragments of the viral EBNA-3, EBNA-4, and EBNA-6 genes and the spliced 131-bp cDNA fragment of the cellular β_2-microglobulin gene (β2-M) were amplified by PCR with intron-flanking primers. As a control, PCR amplification of the genomic B95.8 virus DNA (DNA) was used. The positions of unspliced and spliced PCR products are indicated. (B) The cytoplasmic RNA was fractionated on a 10 to 50% sucrose gradient in the absence (left) or presence (right) of EDTA. Ten fractions were collected (fraction 1 represents the bottom of the gradient, and fraction 10 represents the top), and RNA present in each of these fractions was analyzed by RT-PCR. (C) The A_{260} of each gradient fraction was measured, and the fractions containing intact polysomes are indicated.

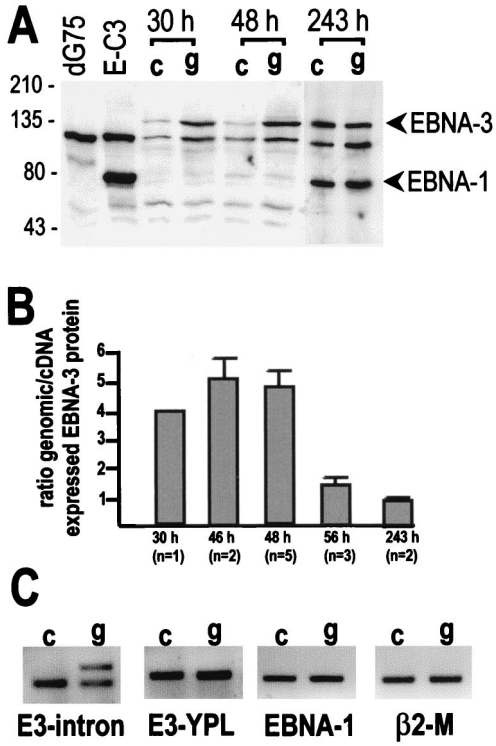

FIG. 8. The intron influences EBNA-3 protein expression. (A) DG75 cells transfected with the cDNA (c) or the genomic (g) form of EBNA-3 were harvested after 30, 48, or 243 h (stable selection). DG75, parental cell line; E-C3, stably selected control vector-transfected DG75 cells. The cell extracts were analyzed by immunoblotting with an EBV-positive human serum. The positions of the EBNA proteins are indicated by arrowheads, and size markers (in kilodaltons) are shown on the left. (B) Histogram of the densitometrically determined immunoblot signals of EBNA-3 protein shown as a ratio of genomic to cDNA expressed EBNA-3 protein. Times of cell harvesting, number of experiments (*n*), and error bars are indicated. (C) Total RNA of cells transfected for 48 h with the cDNA (c) or genomic (g) form of EBNA-3 were reverse transcribed with an oligo(dT) primer. The intron-flanking region (E3-intron) and the second exon (E3-YPL) of EBNA-3 (for primers, refer to Fig. 1), as well as the coding regions of EBNA-1 and β_2-microglobulin (β2-M), were PCR amplified from the first-strand cDNAs. The PCR products were separated on an agarose gel and visualized by ethidium bromide staining.

mRNAs of EBNA-3, EBNA-4, and EBNA-6 were associated with the polysomes. An identical assay was performed in the presence of EDTA, which would disrupt the polysome structure. The sedimentation profile of the EBNA 3 family mRNAs (which were almost exclusively unspliced) correlated well with that obtained from the cellular β_2-microglobulin mRNA, with a significant proportion of the message sedimenting along with the polysomes (Fig. 7B, bottom fractions). Disruption of the intact polysomes by addition of EDTA resulted in a significant proportion of the EBNA-3, EBNA-4, and EBNA-6 unspliced mRNA and β_2-microglobulin spliced mRNA being displaced toward the top of the gradient (Fig. 7B). The A_{260} of the gradient fractions demonstrated that the presence of EDTA resulted in the disruption of the polysomes (Fig. 7C). This data demonstrated that the unspliced mRNAs of the EBNA 3 family genes were indeed transported into the cytoplasm and that a significant proportion of this mRNA was physically associated with polysomes.

No EBNA-3 splice variant proteins are expressed. The association of intron-containing EBNA-3 mRNA with the polysomes suggested that different forms of EBNA-3 protein might be expressed. Since the EBNA-3 intron contained stop codons in all three reading frames and the two EBNA-3 exons are translated in different reading frames, nonremoval of the intron could result in different EBNA-3 isoforms (see Fig. 9). To investigate this possibility, the 8-aa FLAG immunoepitope was introduced into exon 1 and the FLAG-tagged genomic EBNA-3 sequence was stably expressed in DG75 cells. However, besides the full-length epitope tagged EBNA-3 protein, no other FLAG epitope-containing protein form could be detected in immunoblots. In addition, no other protein forms (besides the full-length EBNA-3 protein) could be detected in immunoblots when an EBV-seropositive serum was used (Fig. 8A and data not shown). These results suggested that intron retention in EBNA-3 mRNA did not lead to different isoforms of EBNA-3.

Intron retention influences EBNA-3 protein expression. The high levels of intron-containing EBNA-3 mRNA in the cytoplasm suggested that the unspliced mRNA might influence the level of protein expression. This was addressed by comparing the amount of EBNA-3 protein expressed from either the genomic or the cDNA form of the EBNA-3 gene. Both forms of the EBNA-3 gene were cloned into expression vector EBO-pLPP and were transfected into DG75 cells. Transient cell transfectants were harvested 30 to 48 h after electroporation, and protein expression was analyzed by immunoblotting with a human serum directed against the EBNA proteins (Fig. 8A). Surprisingly, the level of EBNA-3 protein expression was higher in cells containing the genomic form of the EBNA-3 gene. The parallel detection of the level of EBNA-1 protein (derived from the expression vector) confirmed that similar levels of plasmid were present in the cells. Trypan blue cell staining demonstrated that the viability of each of the transfectants was similar. Protein extracts of the parental line DG75 and a vector-only transfected cell line were used as controls (Fig. 8A). The differences in EBNA-3 protein expression were

reproducible in seven independent transient transfections performed with a range of 10 to 25 μg of each plasmid DNA. Within 48 h posttransfection, four- to sixfold more EBNA-3 protein was present in cells containing the genomic construct than in cells containing the cDNA construct (Fig. 8B). At 56 h postelectroporation, EBNA-3 protein expression, between these two constructs, was less pronounced. Following stable selection of cell bulk cultures (243 h), the levels of EBNA-3 protein expression were similar in cell transfectants expressing either the genomic form or the cDNA form of EBNA-3 (Fig. 8A and B). This data indicated that the presence of the intron may enhance the initial levels of EBNA-3 protein expression.

The increase in protein expression could be due to the EBNA-3 intron acting as a transcriptional activator. To address this possibility, the mRNA steady-state levels in cells transiently expressing either the genomic or cDNA form of EBNA-3 were analyzed by RT-PCR (Fig. 8C). Amplification of the second exon of EBNA-3 demonstrated similar amounts of EBNA-3 mRNA in transfectants containing either the genomic form or the cDNA form of EBNA-3. As expected, the EBNA-3 intronic sequence was retained in the transfectants containing the genomic construct but not in cells containing the cDNA construct. The control RT-PCR amplification of EBNA-1 indicated similar levels of expression plasmids in both types of transfectants, while amplification of β_2-microglobulin demonstrated similar amounts of cellular RNA. This data indicated that the presence of the EBNA-3 intron did not enhance transcriptional activity but, rather, suggested that the unspliced EBNA-3 mRNA, present in the cytoplasm, increased protein expression.

DISCUSSION

The data presented in this report indicates for the first time that the bulk of polyadenylated transcripts of the viral EBNA-3, EBNA-4, and EBNA-6 genes contain intronic sequences which are transported into the cytoplasm and associate with polysomes. Intron retention occurred in both transfected BL cells and EBV-positive BL cells and LCLs, indicating that this represents the normal processing of EBNA-3, EBNA-4, and EBNA-6 mRNA. Studies of the EBNA-3 gene by using the S1 nuclease protection assay and Northern blot analyses confirmed that the vast majority of polyadenylated EBNA-3 transcripts contain intron sequence. Since the size of these transcripts is virtually identical to that of the fully spliced mRNA, it is understandable that they have not been detected in previous studies in which probes specific for the coding region of EBNA-3 were used (15, 34).

In mammalian cells, pre-mRNAs are usually processed in a highly coordinated fashion involving formation of a cap at the 5′ end, excision of introns, and polyadenylation of the 3′ end. A model for selection of splice sites (exon definition model) in which exons and not introns are the basic units recognized by the splicing machinery has been proposed (29). First exons are defined by interactions between factors which recognize the 5′ cap and the 5′ splice donor site. Terminal exons are defined by interactions between factors recognizing both the 3′ splice acceptor sites and polyadenylation sites (reviewed in reference 5). Our data demonstrate that intron retention occurs within the EBNA 3 family transcripts derived from three different promoters (EBV, SV40, and RSV), suggesting that it is independent of promoter usage. Neither the full-length EBNA-3 gene nor the coordinate expression of all of the three EBNA 3 family genes was needed for intron retention, suggesting that this feature is intrinsic to the structure of each of these genes.

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       mag GURAGT                          *
EBNA-3 acg GUGAG.CAUCCCUAUGGCCUAAGUGUGUGAUGUGUGUUUUUACCCAUC
EBNA-4 uug GUAAGAGGCACCU..........AGAACAUUUCCAGAUGUUUCGCUU
EBNA-6 aag GUGAG.UAUGCCUCU..............AACUGGGUUCAUGGGGGCC
Consen --g GU-AG-----CCU---------------A--U-------U-------

                                                (Yn)NCAG g
EBNA-3 ACACAACAACAAGGUAAGUAAUUUGUUGCCGUUGGUUUCAG cgc
EBNA-4 GGAUUUUUUGGCCAGUCUUAAUUGAUUGUCAUUGGUUUCAG caa
EBNA-6 AUCUAAGGCCCACGUGUGACCCAUGUUUCCAUUAAUUUUAG caa
Consen ------------------------UU--C-UU--UUU-AG c--
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FIG. 9. Intron sequences of the EBNA 3 family genes. The aligned exon (lowercase) and intron (capital) RNA sequences of EBNA-3, EBNA-4, and EBNA-6 (B95.8 strain [3]) and their consensus sequence are presented. Dots and dashes represent gaps; the asterisk indicates the potential branch point. The mammalian splice donor and acceptor consensus sequences are given in boldface type. Note that the first and second exons of EBNA-3 and EBNA-6 undergo a frameshift after splicing.

Sequence analysis of the introns and their flanking regions showed that they did not have perfect homology to the consensus mammalian splice signals (Fig. 9). Suboptimal 5′ donor and 3′ acceptor splice sites can reduce the efficiency of splicing, leading to the retention of introns, as seen for the bovine growth hormone (10). This premise is supported in the "exon definition model," which requires weak splicing signals for differential splicing to occur (29). Recent data from polyomavirus late mRNA studies demonstrated that a suboptimal 5′ splice site leads to accumulation of unspliced mRNA, which is able to enter the cytoplasm (16). These authors suggested that the process of splicing was not necessary for mRNA export into the cytoplasm. Splicing of introns can be influenced by *cis*- or *trans*-acting mechanisms. Exonic splicing enhancers most commonly promote removal of the flanking upstream intron by interacting with cellular splicing factors (17). Alternatively, exon sequences which repress splicing have been described, particularly if the exons carry weak splice sites (9). The EBNA-3 deletion constructs indicated that an exonic splicing-inhibitory element was probably not involved in intron retention in EBNA-3 mRNA. Although loss of most of the second EBNA-3 exon resulted in predominantly spliced EBNA-3 mRNA, the addition of any EBNA-3 RNA after the 3′ acceptor site of the EBNA-3 intron facilitated intron retention. One possible explanation for this phenomenon is that the additional RNA may be stabilizing some secondary structure within the mRNA, thereby inhibiting splicing. Sequences within an intron can reduce the efficiency of intron removal. In viruses like minute virus of mice or RSV, intronic sequences inhibit the splicing of introns, resulting in a significant portion of unspliced, polyadenylated viral RNA in infected cells (2, 46). Secondary RNA structures within the intron have been reported to control alternative splice site selection for the generation of isoforms of the human growth hormone (12). It is of interest that analysis of minimal free energy release predicted an RNA stem-loop within the EBNA-3 intron, suggesting that this structure might be involved in intron retention.

The EBV genome and the vectors used to express the genes of the EBNA 3 family always expressed EBNA-1, which is necessary for the replication of the episome. EBNA-1 is also known to bind RNA via RGG binding motifs located in the N-terminal half of the protein (37), suggesting that EBNA-1 might be involved in the process of intron retention. However, deletion of most of the EBNA-1 (resulting in the loss of RNA binding [37]) in the EBNA-3 expression vector did not affect the level of unspliced EBNA-3 mRNA. From studies of developmental genes of *Drosophila* (e.g., sxl, tra and dsx), it is known that the proteins, expressed from mRNA in which intron retention or exon skipping occurs, can regulate their own expres-

sion (reviewed in reference 36). However, data from the frameshifted EBNA-3 mutant (resulting in EBNA-3 mRNA but not protein expression) showed that the EBNA-3 protein did not influence the splicing of its own mRNA. This indicated that EBNA-1 and EBNA-3 were not likely to be involved in intron retention within EBNA-3 mRNA but, rather, that cellular factors were probably involved.

Although constitutive splicing of intronic sequences from RNA is the dominant form of gene expression, intron retention, as a form of alternative splicing, has been observed to occur in a number of different genes. Examples of intron retention have been found in the transcripts encoding fibronectin, platelet-derived growth factor A chain, and bovine growth hormone and during the developmental control of *Drosophila* genes. Viruses, such as influenza virus or RSV, produce primary transcripts used in both spliced and unspliced forms to produce different functional proteins (36). Proteins expressed from either the first or second exon of the EBNAs may have functions distinct from the full-length gene products. However, the immunoblot analysis with FLAG-tagged or wild-type EBNA-3 gave no evidence for the existence of different protein isoforms of EBNA-3.

Retained introns can introduce stop codons in the open reading frame or frameshifts, both of which can lead to premature termination of translation. Thus, intron retention can regulate gene expression without changing transcriptional activity. Premature termination of translation of a transcript is almost as economical as regulating initiation of transcription, since most of the expense in expressing a gene occurs at the translational level (6). This is particularly true if the transcript is very large and the first exon is small, as is the case for the EBNA 3 family genes. Each of the introns within the EBNA 3 gene family contains stop codons in all three reading frames, as well a frameshift is required for EBNA-3 and EBNA-6 protein expression. Hence, the presence of the intron would effectively disrupt the translation process. In this context, a recent paper reports that an intron-containing mRNA was associated with polysomes but was not translated unless the intron was removed (7). This intron was both necessary and sufficient to prevent complete translation of polysome-associated mRNA, and this suggested a novel process by which translation of a mRNA could be attenuated. In light of this, intron retention in the EBNA 3 family mRNAs might serve a similar function in the regulation of translation of these mRNAs. In contrast, it was surprising that the transfection data indicated that retention of the EBNA-3 intron could enhance protein expression, since up to sixfold more EBNA-3 protein was detected in transiently transfected cells expressing the genomic form of EBNA-3 than in those expressing the cDNA form. Interestingly, when transient and stable transfectants were compared, the level of genomic expressed EBNA-3 protein did not increase during the hygromycin selection of stable EBNA-3 cell transfectants. This could indicate that there is a maximal limit on the level of EBNA-3 protein tolerable by the cell and this level was already reached during transient transfection. Thus subsequent increase in the level of EBNA-3 protein might be prevented by regulatory factors within the cell. Alternatively, cells overexpressing EBNA-3 may have been negatively selected, as seen for EBV-encoded LMP-1, which is toxic when overexpressed (14). In contrast, the initially low expression levels of EBNA-3 protein steadily increased between 30 and 243 h posttransfection in cells containing the EBNA-3 cDNA. Finally, the cDNA EBNA-3 stable cell transfectants expressed similar amounts of protein to the cells containing the genomic EBNA-3. Assuming a 10% transfection efficiency for the EBNA-3 expression vectors (as judged by a green fluorescent protein reporter plasmid), the initial minor fraction of cells containing the EBNA-3 cDNA expanded during the process of stable hygromycin selection, leading to an overall increase of EBNA-3 protein levels. Up to fivefold-higher levels of protein and RNA expression from genomic intron-containing DNA, relative to cDNA, have been reported previously for a *Drosophila* gene (26). The authors claimed that the increased level of expression was due to higher levels of transcription mediated by an intron enhancer. In contrast, our RT-PCR data measuring the steady-state levels of the transiently expressed EBNA-3 mRNA indicated that the transcription rates for the genomic and cDNA-derived EBNA-3 gene were similar. One possible explanation is that the EBNA-3 intron did not influence the transcription rate of the expression plasmid but, rather, affected the stability, transport, or translation of the RNA. Increased levels of EBNA-3 mRNA might be transported to the cytoplasm in the cells expressing the genomic EBNA-3 construct compared to those in the intronless construct. However, retaining the intron in the EBNA-3 mRNA seems to help increase the overall expression efficiency, and the association of unspliced EBNA-3 mRNA with the polysomes implies an influence on protein translation.

It is intriguing that the introns of the EBNA-3 family genes are strongly conserved between the A and B types of EBV, which are otherwise characterized by pronounced differences in the coding regions of these genes. This selective conversation strongly implies that the introns serve an important function. Despite the conservation of the introns between the two types of EBV, the introns of EBNA-3, EBNA-4, and EBNA-6 do not have significant homology (Fig. 9). Except for the splicing signals (donor and acceptor sites, branch point, and polypyridine tract), the introns have no consensus sequence, suggesting that the mechanism of intron retention in the EBNA-3, EBNA-4, and EBNA-6 mRNAs would be different. This could lead to individual regulation of the expression of each of these genes. Indeed, as all three gene products seem to directly compete with the cellular transcription factor RBP-Jκ/2N, which modulates viral and cellular gene expression (31, 42), one has to postulate that their protein expression must be individually regulated. This could be achieved by cellular factors which interact with different elements in the introns and regulate the levels of intron retention. Since the EBNA 3 family proteins are essential for transformation and survival of EBV-infected B cells (35, 40), EBV must maintain adequate control over their expression. All the EBNA RNAs are encoded from a large primary transcript initiated from the viral Cp or Wp promoter. Hence, altering the rates of transcription from these promoters would result in the regulation not of individual EBNA genes but of the whole set of genes. In contrast, intron retention might provide a means of individually controlling the expression of each of the EBNA-3, EBNA-4, and EBNA-6 proteins.

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